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20-Hydroxyecdysone Accelerates the Flow of Cells into the G_1 Phase and the S Phase in a Male Accessory Gland of the Mealworm Pupa (*Tenebrio molitor*)

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The cells of the bean-shaped accessory glands of mealworms proliferate through the first 7 days of the 9-day pupal stage. Immediately after larval-pupal ecdysis, 25–27% of the cells were in the G_1 phase, 60–65% were in the G_2 phase, and the balance were in S phase. Over the first 4 days of normal development, the S fraction gradually increased, to reach its highest level in the mid-pupa at the time of the major ecdysteroid peak (Delbecque *et al.*, 1978). Thereafter, the S fraction declined until over 95% of the cells had accumulated in G_2 on Day 8. When 0-day pupal glands were explanted into Landureau's S-20 medium for 6 days, the G_1 fraction remained fairly constant (25–30%) while S and the G_2 fractions fluctuated. On the first day *in vitro*, the G_2 fraction declined and the S fraction rose. On the second day in basal media, the S fraction fell and G_2 rose correspondingly until 70% of the cells reached G_2 when cycling stopped on the third day. With addition of 20-hydroxyecdysone to 0-day cultures, the S fraction increased quite sharply. It remained large for all 6 days of the experiment in the continuing presence of hormone. A 1-day pulse of hormone produced a transient increase in S. We blocked cell cycling with hydroxyurea in a stathmokinetic experiment and showed that 20-hydroxyecdysone accelerated the flow of cells from the G_2 phase to the G_1 phase by 2.5-fold. An increase in the G_1 fraction was detected within 10 hr of hormone administration and the effect was dose-dependent with an ED_{50} of 5×10^{-7} *M* for 20-hydroxyecdysone. We conclude that 20-hydroxyecdysone acts at a control point in the G_2 phase. Incubation of the glands with 20-hydroxyecdysone for only 30–60 min followed by washout stimulated the flow from G_2 to G_1 and the effect persisted after transfer of the tissues to hormone-free media. Dose-dependent stimulation also occurred with ponasterone A (ED_{50} 3×10^{-9}) but not with cholesterol.

INTRODUCTION

The insect molting hormone, 20-hydroxyecdysone, accelerates or retards cell cycles; the nature of the effect depends on the target tissue and the hormone concentration. During pupal development, an arrest of the cell cycle in the G_2 phase has been correlated with rising levels of ecdysteroids in the sternal epidermis of *Tenebrio molitor* (Besson-Lavoignet and Delachambre, 1981) and in the imaginal discs of *Drosophila melanogaster* (Graves and Schubiger, 1982; Fain and Stevens, 1982). In the K_c cell line from *D. melanogaster*, arrest in G_2 follows administration of ecdysteroids *in vitro* (Courgeon, 1972; Stevens *et al.*, 1980). In contrast, enhanced mitotic activity follows after administration of ecdysteroids *in vitro* to locust spermatogonia (Dumser, 1980), mealworm male accessory glands (Szopa *et al.*, 1985) and *Manduca* epidermis (Kato and Riddiford, 1987). The present paper concerns ecdysteroid action during the cell cycle in the mealworm accessory gland.

Male accessory glands of *T. molitor* comprise the paired bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs) (Happ, 1984). BAGs and TAGs are derived from a common mesodermal rudiment (Huet, 1966) that grows by cell proliferation in the pupa and differentiates in the adult. During the first 6 days of the pupal stage, mitotic rates are high in the TAGs (Happ and Happ, 1982) and the BAGs (Grimes and Happ, 1980; Happ *et al.*, 1985) through the very large mid-pupal peak of ecdysteroid (Delachambre *et al.*, 1980). In contrast, for the cells of the sternal epidermis, the beginning of this same pupal ecdysteroid peak is correlated with a blockage of cell cycling (Besson-Laviognet and Delachambre, 1981). Arrest occurs later for the secretory cells of the male accessory glands; mitoses in the BAGs and the TAGs cease in the late pupal stage (Happ *et al.*, 1985) as ecdysteroid levels fall (Delachambre *et al.*, 1980). At least in the TAGs, most cells arrest in G_2 (Happ *et al.*, 1985).

In a previous study, we showed that addition of 20-hydroxyecdysone to organ cultures increased the mitotic index within BAGs and TAGs (Szopa *et al.*, 1985). To explore the effects of this hormone on each phase of the cell cycle, we applied 20-hydroxyecdysone *in vitro* to cultured BAGs under conditions when the cells were

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allowed to cycle freely and under conditions when the cells were blocked in S with hydroxyurea. We show that the hormone affects the flow of cells through mitosis by acting in G_2 and through the S phase by acting at an unknown point.

MATERIALS AND METHODS

Animals. Larvae of *T. molitor* were purchased from a commerical supplier and reared on a diet of Purina Chick lab chow at 25°C. After pupation, white pupae (0–4 hr after ecdysis) were sexed and males were kept at 25°C (Szopa *et al.*, 1985).

In vitro culture. Landureau S-20 culture medium was prepared according to Szopa *et al.* (1985), except that vitamin concentrations were doubled and the pH was 6.9. All procedures were carried out in a laminar-flow hood using standard aseptic techniques. BAG/TAG complexes were dissected from the pupae in S-20 medium. About eight gland complexes were cultured at 25°C in a small petri dish containing 3.0 ml of medium. Media and all solutions of inhibitors and hormones were filter sterilized (0.22 μm, Gelman).

In some experiments, hormone was removed from the cultures. Medium was carefully pipetted off the cultures

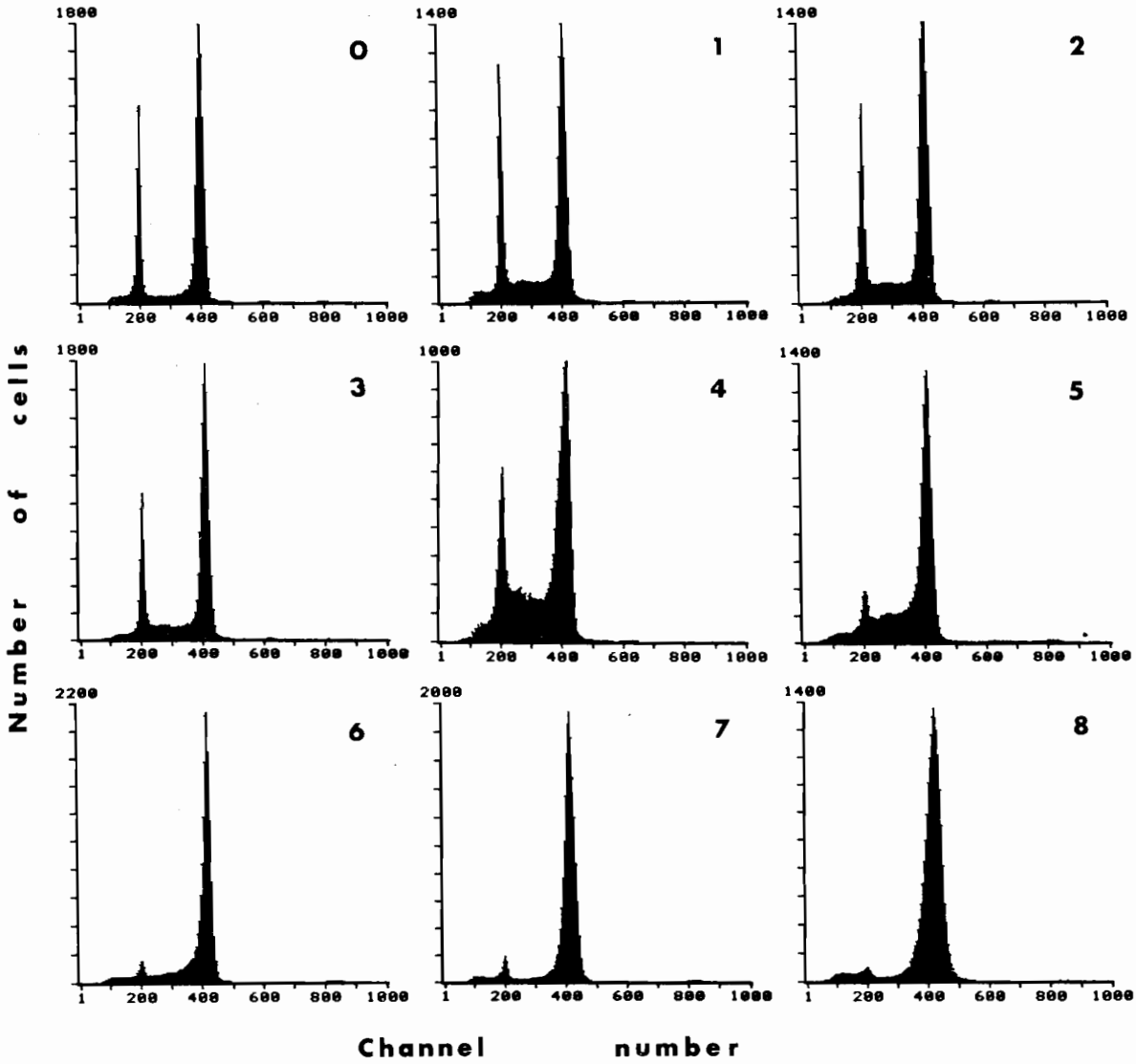

FIG. 1. Changes in percentage of BAG cells in G_1, S, and G_2 phases during the pupal stage. Glands were dissected from pupae and analyzed immediately. The histograms show the distribution of nuclei with DNA contents determined by their propidium iodide fluorescence. Channel number indicates DNA content per nucleus; channel numbers 200 and 400 represent nuclei in G_1 and G_2, respectively. Intermediate DNA channels between the G_1 and G_2 peaks are cells in S. The numbers from 0 to 8 on the right upper corner of each panel indicate pupal age in days. For each day, glands from eight pairs of BAGs were pooled for isolation of nuclei. Nuclei have been stained with propidium iodide as described under Materials and Methods.

and 3.0 ml of new medium was introduced. This procedure was repeated five times with hormone-free media.

Flow cytometric analysis. BAGs were separated from TAGs in 40 m*M* citrate–NaOH buffer (pH 7.6) containing 250 m*M* sucrose and 5% dimethyl sulfoxide (citrate buffer). The nuclear fraction was isolated and stained with propidium iodide according to Happ *et al.* (1985). The stock solution contained 3.4 m*M* trisodium citrate, 0.5 m*M* Tris, and 0.1% Nonidet-P40, adjusted with 1 *N* NaOH to pH 7.6. Pancreatic ribonuclease (1 mg/ml, Sigma) or propidium iodide (0.42 mg/ml, Sigma) was dissolved in the stock solution and aliquots were frozen at −20°C until used. Dissected BAGs (usually eight pairs) were placed in a 1-ml disposable syringe within a 3-ml disposable culture tube in an ice bucket. A 23-gauge needle was attached to the syringe; 50 μl of citrate buffer was added, and BAG cells were dispersed by five passages through the needle. This fresh cell suspension was mixed with 450 μl of stock solution by three passages through the needle in a 10-min period. An aliquot (375 μl) of ribonuclease solution was added and the whole mixture was passed through the needle three times for another 10 min. Finally, 375 μl of propidium iodide solution was added and mixed. The sample was filtered through a 20-μm nylon mesh (Tetko Corp.) and kept on ice for 2–3 hr until analysis (Happ *et al.*, 1985).

The nuclei in this filtrate were subjected to flow cytometry on an Ortho Model 50H Dual-laser cytofluorograph (FACS) with a 2150 data handling system. The proportion of nuclei in each phase of the cell cycle was determined from total counts of nuclei using a polynomial iterative model function (Gray *et al.*, 1979) in a computer program supplied by Ortho. The error is usually +3%. It should be noted that only membrane-bound nuclei are counted so that metaphase, anaphase, and early telophase cells are likely to be lost from the analysis since these stages lack a nuclear membrane. Prophase cells are scored as G_2. In BAGs the maximum mitotic index is 8–9% (Szopa *et al.*, 1985) and thus there is a potential overestimate of up to 4.5% ($\frac{1}{2}$ *M* fraction) in our G_2 determinations.

Hormones. 20-Hydroxyecdysone (Calbiochem) and ponasterone A (a gift from Dr. J. D. O'Connor, UCLA) were dissolved in 10% isopropanol and methanol, respectively. The concentrations were checked spectrophotometrically at 240 nm (E_{240} = 12,670) for 20-hydroxyecdysone and at 244 nm (E_{244} = 12,400) for ponasterone A. Each stock solution was added to S-20 medium to give the desired hormone concentration.

RESULTS

BAG Cells Arrest in the G_2 Phase Late in the Pupal Stage

BAGs were dissected from 0-day pupae and at each day thereafter until the eighth day (1 day before adult ecdysis). Each nuclear fraction was isolated and the percentages of cells in G_1, S, and G_2 phases were determined by flow cytometry (Fig. 1).

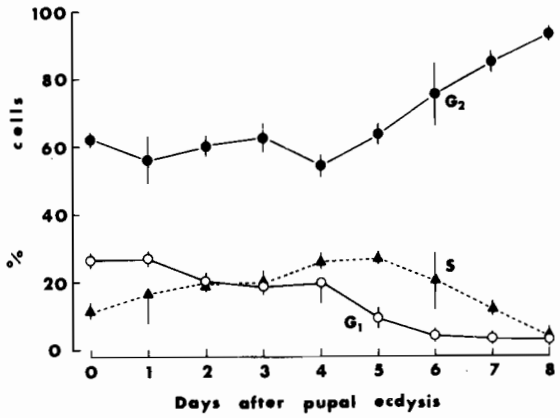

FIG. 2. Percentages of cells in G_1, S, and G_2 phases, calculated from data in Fig. 1 using a polynomial iterative model (see Materials and Methods). Each point represents a mean of duplicate experiments ± SEM.

Just after pupal ecdysis, the percentage of cells in the G_2 phase (>60%) was over twice that in the G_1 phase (25–30%); less than 12% of the cells were in S phase (Figs. 1, 2). The S fraction increased steadily to reach a peak of 25% on the fourth pupal day. After 4 pupal days, the G_2 fraction rose sharply, first apparently at the expense of the G_1 fraction and then also due to depletion of the S fraction, until the eighth day when 97% of the cells of the BAG had reached G_2 and cell cycling had stopped (Fig. 2).

At the beginning of the pupal stage when the S fraction is increasing, ecdysteroid levels are low. The S fraction stays large through the mid-pupal ecdysteroid peak (Delachambre *et al.*, 1980) and then declines as the cells accumulate in the G_2 (Figs. 1, 2). These correlations suggest that the fall of 20-hydroxyecdysone induced the arrest of cells in G_2. To explore this possibility, we applied hormone to glands cultured *in vitro*.

20-Hydroxyecdysone Accelerates Cell Cycling in Vitro

For experiments on the effect of hormone *in vitro*, we used 0-day pupal BAGs because they had not yet been exposed to the pupal ecdysteroid peak *in vivo* (Delachambre *et al.*, 1980). After dissection from the pupae, the BAG/TAG complexes were placed in Landureau's S-20. In control cultures, the glands were maintained for 6 days in basal medium. Two experimental culture groups were exposed to physiological peak levels of 20-hydroxyecdysone (10^{-5} *M*). In the first group (continuous exposure), glands were exposed to hormone throughout the 6-day incubation period. In the second group (hormone pulse), glands were exposed to hormone for only the first day *in vitro*. After washout of

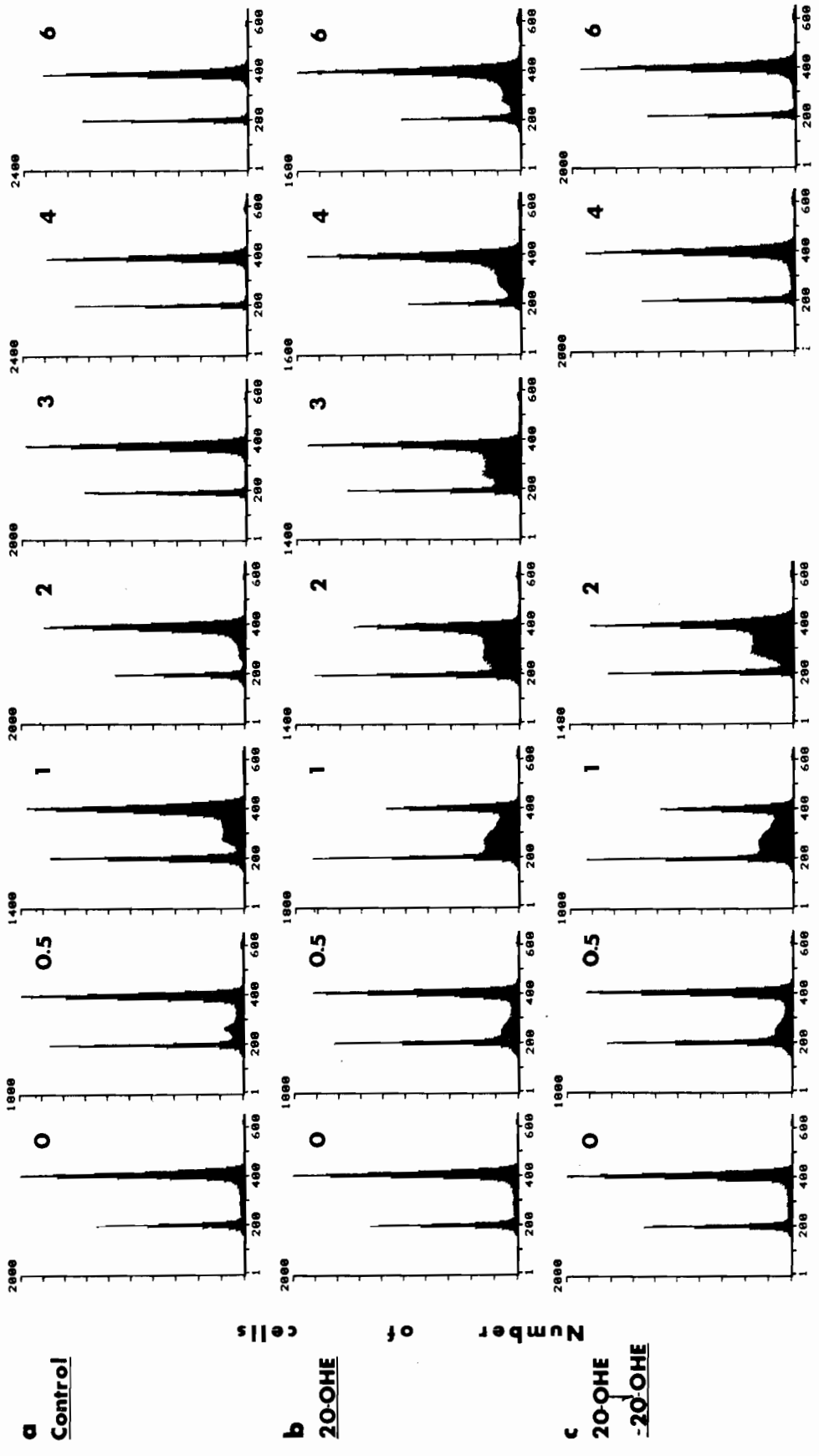

FIG. 3. Changes in the percentages of cells in G_1, S, and G_2 phases after culturing of 0-day pupal BAGs in hormone-free media or in media containing 20-hydroxyecdysone for varying periods. (a) Controls: BAGs incubated continuously in hormone-free S-20 medium. (b) BAGs incubated continuously in medium containing 10^{-5} *M* 20-hydroxyecdysone (20-OHE). (c) BAGs incubated for 1 day in medium with 10^{-5} *M* 20-hydroxyecdysone and then transferred to hormone-free medium (20-OHE → −20-OHE). The numbers from 0 to 6 in the upper right corners of each panel represent days after incubation. After indicated times in culture, BAGs were dissected from adhering TAGs, and the nuclei were isolated, stained with propidium iodide, and subjected to flow cytometry.

the hormone, glands in the hormone pulse group were maintained in basal medium for 5 more days. In control and experimental groups, BAGs were isolated for flow cytometry after 0.5 day in culture and at daily intervals (Fig. 3). Replenishment of the medium after 1 day of culture did not accelerate cell cycling but occasionally reduced it, aparently due to mechanical damage.

Certain features of the results were common to all culture situations. At the end of the first day of culture, the S fraction had increased and the G_2 fraction had declined (Fig. 4). The G_1 fraction remained between 20 and 30% throughout the 6 days. Whenever arrest occurred *in vitro* (Figs. 3a and 3c), 25–30% of the cells remained in G_1 with the balance in G_2 (Figs. 4a and 4c), in contrast to normal development *in vivo* when almost all cells arrested in G_2 (Figs. 1 and 2).

After 12 hr of culture in basal medium, the cells in the S phase were distributed in a symmetrical peak in the early S phase (Fig. 3a). Twelve hours later, the total S fraction was still larger (Fig. 4a) but the early S population was depleted (Fig. 3a). After 3 days, no significant fraction of cells remained in S; 27% were in G_1 and 72% were in G_2.

Whenever hormone was present in the culture media, a significant fraction of cells was found in the S phase (Figs. 3b and 4b). Over the first day, the G_2 fraction declined dramatically from 75 to 38%, while the population in early S increased correspondingly (Fig. 3b). When hormone was left in the medium, the total S fraction rose even higher (almost 40%) by 2 days (Fig. 4b). Between 2 and 6 days, the total S fraction fell gradually with a noticable depletion of the early S component as the G_2 fraction rose (Fig. 4b). Even at 6 days, 23% of the cells persisted in late S (Figs. 3b and 4b).

After a hormone pulse for 1 day was followed by washout, the total S fraction (and especially its early component) declined much more rapidly than with continuous presence of hormone (compare Figs. 3c and 4c with Figs. 3b and 4b) until only 5% of the cells appeared to remain in S after 6 days of culture.

Since 20-hydroxyecdysone accentuated both the fall in the G_2 fraction and the rise in the S fraction, we conclude that the hormone accelerated the flow of cells into G_1 and into S.

20-Hydroxyecdysone Stimulates Flow of Cells from G_2 to G_1 in the Presence of Hydroxyurea

To estimate the rate of movement of cells from the G_2 phase into the G_1 phase, we employed a stathmokinetic experimental design patterned after that of Puck and Steffen (1963) who blocked mitosis with colchicine. In the present case, we used hydroxurea as the agent to arrest cells near the G_1-S boundary (Prescott, 1976). The fraction of cells accumulating in the G_1 phase upstream of the block was plotted as $\ln(1 + G_1)$ versus the incubation time, and from the slope of the regression line, we calculated the rate of movement from G_2 to G_1. In preliminary experiments, 5 m*M* hydroxyurea was found to be the maximum concentration which allowed significant movement of cells from G_2 to G_1 and did not produce extensive cell death.

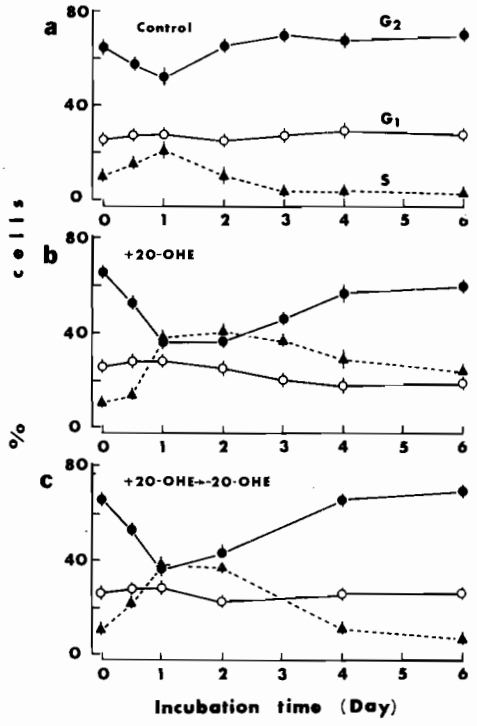

FIG. 4. Percentages of cells in G_1, S, and G_2 phases calculated from data in Fig. 3. (a) BAGs incubated continuously in hormone-free S-20 medium (control). (b) BAGs incubated continuously in medium containing 10^{-5} *M* 20-hydroxyecdysone (+20-OHE). (c) BAGs incubated for 1 day in medium with 10^{-5} *M* 20-hydroxyecdysone and than transferred to hormone-free medium (+20-OHE → −20-OHE). Each point represents a mean of duplicate experiments ± SEM.

Accumulation in G_1 occurred in all cultures containing 5 m*M* hydroxyurea. More cells collected in G_1 within cultures with hormone; therefore the 20-hydroxyecdysone acts after the block, in early S and before G_1. In the logarithmic plot, the accumulation functions were almost straight lines, both for glands exposed to 20-hydroxyecdysone (10^{-5} *M*) and for controls (Fig. 5). The regression lines intersect at about 5 hr after administration of hormone, suggesting that the hormone effect is patent within 5–10 hr. For the period from 5 to 25 hr, the flow of cells into G_1 was found to be 2.5 times faster in media with 20-hydroxyecdysone than in hormone-free media. Using the equations of Kimmel *et al.* (1983), we calculated the doubling time for cells in media with 20-hydroxyecdysone to be 61 hr while that for cells in hormone-free media was 154 hr.

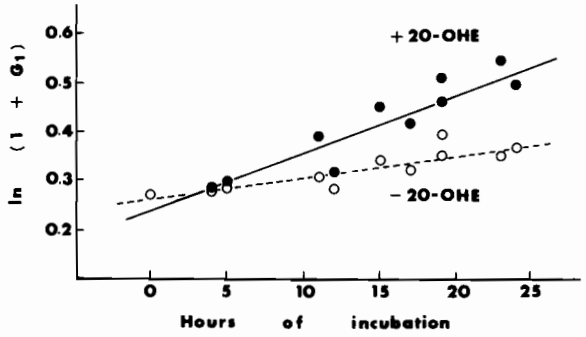

FIG. 5. The rate of cell entrance into the G_1 phase in cultures with and without 20-hydroxyecdysone. BAGs from 0-day pupa were used. All media contained 5 m*M* hydroxyurea. The hormone-treated group (+20-OHE) was cultured with 10^{-5} *M* 20-hydroxyecdysone while the control media (−20-OHE) contained no hormone. After given times, BAGs were separated from TAGs and the nuclear fraction was isolated and stained for flow cytometry. The percentage of cells in the G_1 phase was plotted as $\ln(1 + G_1)$ versus incubation time, according to Kimmel *et al.* (1983). Regression lines have slopes of 0.0114 ($r = 0.943$) and 0.0045 ($r = 0.862$) for 20-hydroxyecdysone-treated and hormone-free media, respectively.

Cell Cycling and Steroid Dose

The stathmokinetic design was used to investigate the relationship between concentrations of 20-hydroxyecdysone, ponasterone A (25-deoxy-20-hydroxyecdysone), or cholesterol upon flow of cells from G_2 into G_1 (Fig. 6). The rate of movement of cells was dependent on the dose of 20-hydroxyecdysone ($ED_{50} = 5 \times 10^{-7}$ *M*) and of ponasterone A ($ED_{50} = 3 \times 10^{-9}$ *M*). High levels of cholesterol (2×10^{-6} *M*) produced no acceleration of cell cycling.

How Long Must 20-Hydroxyecdysone be Present to Stimulate Cycling?

To determine the shortest duration of hormone exposure required for the acceleration of the cell cycle, we exposed BAGs to hormone for increasingly longer periods of time. In all cases, the interval between dissection of glands from the pupae and the end of the incubation *in vitro* was 24 hr and hydroxyurea was included in the culture media. Following exposure of BAG/TAG complexes to 20-hydroxyecdysone, the hormone was washed out, and the glands were transferred to hormone-free media. After a 20-min exposure to hormone followed by 23 hr 40 min in basal medium, no stimulation was detected (Fig. 7). However, after 30–60 min of exposure to 20-hydroxyecdysone followed by the balance of the day in basal medium, flow rate was accelerated to produce a sustained effect (Fig. 7).

DISCUSSION

Cell Cycling during Normal Development in Vivo

The present cytometric results on changing distribution of cells in G_1, S, and G_2 fractions of the pupal BAGs are consistent with those previously reported. High mitotic rates are seen during the first 5–6 days of the pupal stage but cell division ceases by Day 7 (Grimes and Happ, 1980; Happ and Happ, 1982; Happ *et al.*, 1985).

At the start of the pupal stage when mitoses were not detected in the BAGs (Happ *et al.*, 1985), two-thirds of the cells are in G_2 (Fig. 1). The ensuing period of rapid mitoses is followed by a decline on Days 5 and 6 and by 8

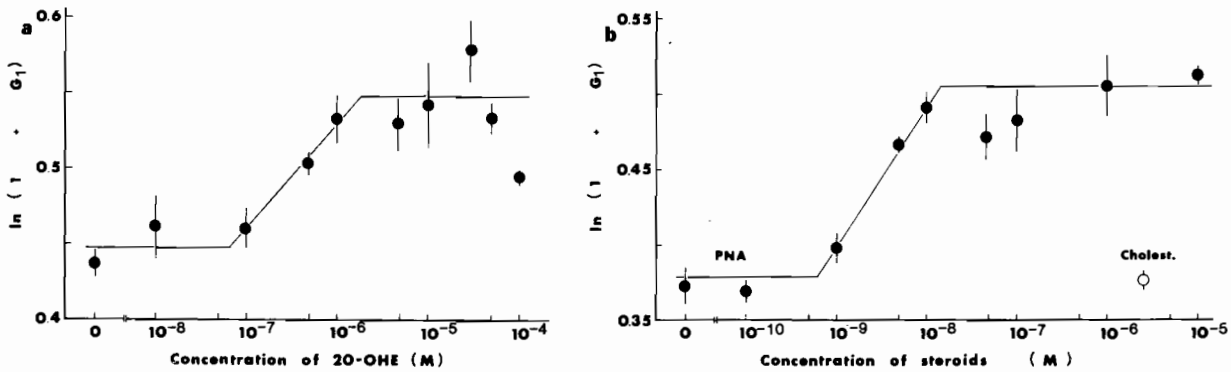

FIG. 6. Dose-response curve for the acceleration of flow of 0-day pupal BAG cells from G_2 into G_1 in media containing 5 m*M* hydroxyurea. (a) 20-Hydroxyecdysone (20-OHE). Three microliters of 10% isopropanol was added to 3 ml medium. (b) Ponasterone A (PNA) or cholesterol (Cholest.) Ninty-six microliters of methanol was added to 3 ml medium. After 24 hr of incubation, BAGs were separated and the nuclear fraction was isolated. The percentage of cells in the G_1 phase was plotted as $\ln(1 + G_1)$. Each point represents the mean of three to four experiments ± SEM.

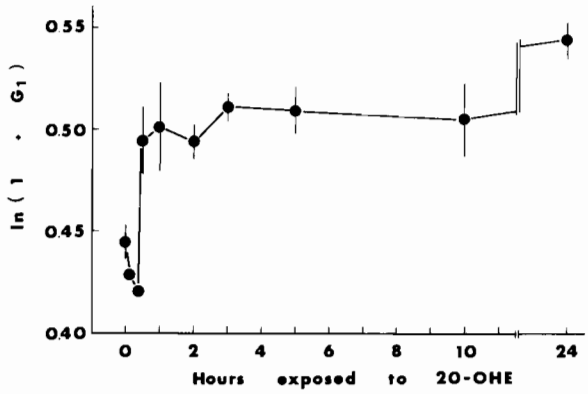

FIG. 7. Effects of duration of hormone exposure on flow of cells from G_2 to G_1. BAG/TAG complexes were dissected from 0-day pupae and incubated in medium containing 5 m*M* hydroxyurea. At first, the medium also contained 20-hydroxyecdysone (10^{-5} *M*), which was washed out at times indicated and the glands were incubated for the balance of the 24-hr experiment in medium without hormone. After a 24-hr incubation, BAGs were separated from TAGs and the nuclear fraction was isolated for propidium iodide staining and flow cytometry. The percentage of cells in the G_1 phase was plotted as $\ln(1 + G_1)$. Each point is the mean of three to eight experiments ± SEM.

days, all the cells are arrested in G_2 (Figs. 1 and 2). As shown in Figs. 1 and 2, movement of cells through G_1 is almost complete by Day 6. However, cells continue to flow through S and to accumulate in G_2 for 2 days longer. The threefold increase in cell number, from 2.4 $\times 10^5$ to 7.5 $\times 10^5$ (Happ *et al.*, 1985), is consistent with one mitosis for all cells that begin the pupal stage in G_1 and S and two mitoses for all cells that are in G_2 just after pupal ecdysis. Since the distribution of cells among G_1, S, and G_2 (Fig. 2) and M (Happ *et al.*, 1985) changes relatively little between Days 1 and 4, the dividing cells do not appear to be in synchrony during this interval of relatively steady growth. The fact that the G_2 fraction is large relative to the G_1 fraction at all times sampled argues for a G_2 phase that is several times longer than the G_1 phase, but a precise calculation of the lengths of these phases is not possible at this time.

Ecdysteroids Act in Vitro at Physiological Doses

The concentration of 20-hydroxyecdysone which accelerated cell cycling ($ED_{50} = 5 \times 10^{-7}$ *M*) (Fig. 6a) is well below the peak level in the mid-pupa (8×10^{-6} *M*) (Delachambre *et al.*, 1980). The hormone produced a lasting effect; a 30- to 60-min exposure to hormone persisted for many hours thereafter (Fig. 7). Ponasterone A was more effective, by two orders of magnitude ($ED_{50} = 3 \times 10^{-9}$ *M*) (Fig. 6b). We believe that this result constitutes the first demonstration that the phytoecdysteroid ponasterone A acts on beetle cells. For *Drosophila* cells, ponasterone A is two orders of magnitude more potent than 20-hydroxyecdysone (Stevens *et al.*, 1980) but ponasterone A has little effect on *Manduca* (J. D. O'Connor, personal communication). Cholesterol had no activity on cell cycling in the present study of the BAGs. In earlier studies, we showed that ecdysone, presumed to be the prohormone, was not nearly as effective as 20-hydroxyecdysone itself in stimulating mitoses (Szopa *et al.*, 1985). The results from the present study using flow cytometry and from the earlier one using mitotic indices (Szopa *et al.*, 1985) indicate that the steroid-produced acceleration of cell cycling is most likely due to a true hormonal action rather than merely a nutritive effect on the cultured organs.

20-Hydroxyecdysone Accelerates Cell Cycling through M and through S Phases in Vitro

Three lines of evidence argue that 20-hydroxyecdysone acts in the G_2 phase. First, the effectiveness of 20-hydroxyecdysone in the presence of hydroxyurea (Fig. 5) shows that the hormone must act between early S and G_1. Second, decline in the G_2 fraction is an early reflection of hormone activity (Fig. 4). The 2.5-fold decrease in doubling time calculated from the regression lines (Fig. 5) is consistent with the threefold faster decline in the G_2 population during the first day *in vitro* when they were allowed to cycle freely (Fig. 4). Third, the lag between hormone administration and its affect is relatively short: the cycling rates of control and hormone-treated cells diverge within 10 hr after explantation (Fig. 5). As noted above, the large G_2 fraction and the long doubling time of several days (Happ *et al.*, 1985) are consistent with a G_2 that lasts much longer than the 5-10 hr required for a detectable effect in the G_1 fraction.

Regulation of the cell cycle in the G_2 phase has been reported from mammalian cells (Gelfant, 1963; Rao, 1980), plant embryos (e.g., Van't Hof *et al.*, 1973), *Hydra* (Herrmann and Berking, 1987), and insects (examples cited under Introduction). Regulation in the G_1 phase is more widely known and has been more extensively studied (e.g., Hartwell, 1974; Pardee, 1974; Smith and Martin, 1973). In some cells, there is regulation in both G_1 and G_2, as in the fission yeasts with genes acting in both G_1 and G_2 (Nasmyth, 1979; Fantes and Nurse, 1977). The yeast genes encode protein kinases (Reed *et al.*, 1985; Simanis and Nurse, 1986) that appear to have human homologs (Draetta *et al.*, 1987). The interspecific differences in the control of cell cycling may merely reflect divergent emphasis during evolution on one or another of an ancestral set of regulatory points.

20-Hydroxyecdysone also affects the S fraction of the

cells of the BAGs. The rise in the S fraction on the first day after explantation was much sharper in the presence of 20-hydroxyecdysone, such that an unphysiologically high fraction of cells entered S (Figs. 3 and 4). On the basis of the present results, we cannot determine whether sustained flow through S is due to hormone acting in the G_1 phase or earlier (in G_2) to speed the flow of cells into and through G_1.

Ecdysteroids and Differentiation in the BAGs

While the cells of the BAGs are dividing in the pupa, they remain undifferentiated (Grimes and Happ, 1980; Dailey and Happ, 1983). At 8–9 pupal days, close to the time of adult ecdysis, adult-specific proteins begin to accumulate (Happ *et al.*, 1982; Happ, 1984; Grimnes and Happ, 1986; Grimnes *et al.*, 1986; Shinbo *et al.*, 1987; Yaginuma and Happ, 1987) and definitive secretory vesicles characteristic of specific adult cell types appear (Dailey and Happ, 1983). Ecdysteroids also play a role in the acquisition of competence to make adult-specific secretory proteins (Grimnes and Happ, 1987; Yaginuma and Happ, 1988).

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